

E3

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 35/16, 37/02	A1	(11) International Publication Number: WO 94/07510 (43) International Publication Date: 14 April 1994 (14.04.94)
(21) International Application Number: PCT/SE93/00793 (22) International Filing Date: 1 October 1993 (01.10.93) (30) Priority data: 9202878-6 2 October 1992 (02.10.92) SE 9301580-8 7 May 1993 (07.05.93) SE 9302006-3 11 June 1993 (11.06.93) SE (71) Applicant (for all designated States except US): KABI PHARMACIA AB [SE/SE]; S-751 82 Uppsala (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): ÖSTERBERG, Thomas [SE/SE]; Folkungagatan 88 B, S-116 22 Stockholm (SE). FATOUROS, Angelica [SE/SE]; Tomtebgatan 35, S-113 38 Stockholm (SE).		(74) Agents: TANNERFELDT, Agneta et al.; Kabi Pharmacia AB, S-112 87 Stockholm (SE). (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE); OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER (57) Abstract The present invention relates to novel composition comprising coagulation factor VIII and a non-ionic surfactant such as block copolymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80 as stabilizer. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars or sugar alcohols. The invention also relates to the use of a non-ionic surfactant as stabilizer for a composition comprising coagulation factor VIII.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TC	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

COMPOSITION COMPRISING COAGULATION FACTOR VIII
5 FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A
SURFACTANT AS STABILIZER.

The present invention relates to a novel formulation comprising coagulation
factor VIII and a non-ionic surfactant such as block co-polymers, e.g.
10 polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g.
polysorbate 20 or polysorbate 80. The composition can also comprise sodium
chloride, calcium chloride, L-histidine and/or sugars and/or sugar alcohols.

Haemophilia is an inherited disease which has been known for centuries but
15 it is only within the last three decades that it has been possible to
differentiate between the various forms; haemophilia A, haemophilia B and
haemophilia C. Haemophilia A is the most frequent form. It affects only
males with an incidence of one or two individuals per 10 000 live-born
males. The disease is caused by strongly decreased level or absence of
20 biologically active coagulation factor VIII (antihæmophilic factor) which is a
protein normally present in plasma. The clinical manifestation of
haemophilia A is a strong bleeding tendency and before treatment with
factor VIII concentrates was introduced, the mean age of those patients was
less than 20 years. Concentrates of factor VIII obtained from plasma have
25 been available for about three decades. This has improved the situation for
treatment of haemophilia patients considerably and given them possibility
to live a normal life.

Therapeutic factor VIII concentrates have until now been prepared by
30 fractionation of plasma. However, there are now methods available for
production of factor VIII in cell culture using recombinant DNA techniques
as reported in e.g. J Gitschier et al. Nature 312, 330-332, 1984 and EP 160 457.

Factor VIII concentrates derived from human plasma contain several
35 fragmented fully active factor VIII forms (Andersson et al, Proc. Natl. Acad.
Sci. USA, Vol 83,2979-83, May 1986). The smallest active form has a

molecular mass of 170 kDa and consists of two chains of 90 kDa and 80 kDa held together by a metal ion bridge. Reference is here made to EP 197 901.

- 5 Kabi Pharmacia has developed a recombinant factor VIII product which corresponds to the 170 kDa plasma factor VIII form in therapeutic factor VIII concentrates. The truncated recombinant factor VIII molecule is termed r-VIII SQ and is produced by Chinese Hamster Ovary (CHO) cells in a cell culture process in serum free medium at finite passage.

- 10 The specific activity of r-VIII SQ could be more than 12 000 IU/mg protein and preferably more than 14 000 IU/ mg. Activity of about 15 000 IU/mg has been measured. About 10 000 IU VIII:C per mg protein has earlier been known for our r-VIII SQ.

- 15 Recombinant factor VIII SQ is indicated for treatment of classical haemophilia. The dosage is similar to the dosage of the plasma factor VIII concentrates. Due to the high concentration now obtainable only small volumes are needed for injection.

- 20 The structure and biochemistry of recombinant factor VIII-products in general have been described by Kaufman Tibtech, Vol 9,1991 and Hematology, 63, 155-65, 1991. The structure and biochemistry of r-VIII SQ have been described in WO 91/09122.

- 25 The stability of proteins is generally a problem in pharmaceutical industry. It has often been solved by drying of the protein in different drying processes, such as freeze drying. The protein has thereafter been distributed and stored in dried form.

- 30 The solution before drying or freeze-drying, the dried material and the reconstituted product should all be stable, so that not too much activity is lost during the drying process, the storage or during handling.

Factor VIII which has been fractionated from plasma is normally sold as lyophilized powder which should be reconstituted with water.

- 35 A formulation with a low amount of protein will generally loose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human

- albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang et al., J. of Parenteral Sci. and Tech. Vol 42, Number 2S, supplement. 1988). Human albumin is
- 5 also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of factor VIII is known and is currently used in all highly purified factor VIII products on the market.
- However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of
- 10 human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.
- 15 There is a need for albumin free formulations containing factor VIII and especially recombinant factor VIII which are stable during drying or freeze-drying, in solution and as a solution after reconstitution.
- Several solutions have been proposed for the stabilization of different
- 20 proteins:
- EP 35 204 (Cutter) discloses a method for imparting thermal stability to a protein composition in the presence of a polyol.
- 25 EP 381 345 (Corint) discloses an aqueous liquid of a peptide, desmopressin, in the presence of carboxymethylcellulose.
- In WO 89/09614 (Genentech), a stabilized formulation of human growth hormone comprising glycine, mannitol and a buffer is disclosed and in a
- 30 preferred embodiment a non-ionic surfactant such as polysorbate 80 is added. The non-ionic surfactant is added for reduced aggregation and denaturation. The formulation has an increased stability in a lyophilized formulation and upon reconstitution.
- 35 EP 268 110 (Cetus) discloses a solution comprising a particular protein, interleukin-2, which is dissolved in an inert carrier medium comprising a non-ionic polymeric detergent as a solubilizer/stabilizer. The preferred

detergents are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds and polyethylene sorbitan fatty acid esters.

5 US 4 783 441 (Hoechst)discloses an aqueous solution comprising a protein, such as insulin and a surface active substance.

10 US 4 165 370 (Coval) discloses a gamma globulin solution and a process for the preparation thereof. The solutions contains polyethylene glycol (PEG). A non-ionic surfactant can be added to the solution.

15 In EP 77 870 (Green Cross) the addition of amino acids, monosaccharides, oligosaccharides or sugar alcohols or hydrocarbon carboxylic acid to improve stability of a solution containing factor VIII is disclosed and the addition of sugar alcohol or disaccharides to an aqueous solution of factor VIII for increasing stability during heat treatment has been disclosed in EP 117 064 (Green Cross).

20 WO 91/10439 (Octopharma) claims stable injectable solution of factor VIII or factor IX which comprises a disaccharide, preferably saccharose and one or more amino acids.

EP 315 968 and EP 314 095 (Rorer) claim stable formulations of factor VIII with different ionic strength.

25 Proteins are different with regard to physico-chemical properties. When preparing a pharmaceutical preparation which should be physico-chemical acceptable, and stable for a long time, consideration can not only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient
30 and safety for the patient. The results of these aspects are not predictable when testing different formulations and there often is a unique solution for each protein.

35 In plasma circulating factor VIII is stabilized by association with its carrier protein, the von Willebrand factor (vWF). In plasma and also in conventional intermediate purity factor VIII concentrates the ratio vWF to factor VIII is at least 50:1 on a weight basis. In very high purity factor VIII

concentrates, with a specific activity of more than 2 000 IU per mg protein, the ratio vWF to factor VIII is about 1:1 (w/w) and essentially all factor VIII is bound to vWF. Despite this stabilization further protection by the addition of albumin is required in order to achieve an acceptable stability during lyophilization and storage.

All super pure preparations on the market are stabilized with albumin (human serum albumin).
There is now a demand for injectable factor VIII without albumin and containing a minimum of additives.

We have now developed a new formulation which solves the above mentioned problems for factor VIII.

To our great surprise we have found that factor VIII, which is a very sensitive protein, can be stabilized without albumin, when a non-ionic surfactant is added.

Thus the present invention relates to a composition comprising a coagulation factor VIII and a non-ionic surfactant as stabilizer. Our factor VIII is highly purified, i.e. has a specific activity of more than 5000 IU/mg protein, and the composition is stabilized without the addition of albumin.

When factor VIII is recombinant it can be either in its full-length form or as a deletion derivative such as SQ derivative.

The amount of factor VIII is from 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.

The non-ionic surfactant is preferably chosen from block co-polymers such as a poloxamer or polyoxyethylene (20) fatty acid ester, such as polysorbate 20 or polysorbate 80. Tween 80® has been used as polysorbate 80.

The non-ionic surfactant should be present in an amount above the critical micelle concentration (CMC). See Wan and Lee, Journal of Pharm Sci, 63, 136, 1974.

The polyoxyethylene (20) fatty acid ester is thus preferably in an amount of at least 0.01 mg/ml. The amount could e.g. be between 0.02 and 1 mg/ml.

The composition can also comprise sodium or potassium chloride, preferably in an amount of more than 0.1 M.

The composition comprises preferably a calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM and an amino acid such as L-histidine in an amount of more than 1 mM.

The amount could e.g. be chosen between 0.05 and 500 mM.

- 5 Mono-or disaccharides such as sucrose or sugar alcohols could be added e.g. in an amount of 1 to 300 mg/ml.

- 10 The composition comprises preferably L-histidine and sucrose. The ratio sodium chloride to L-histidine in the composition is preferably more than 1:1.

The composition could comprise

- i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
15 iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

- 20 To this composition could mono-or disaccharides or sugar alcohols, preferably sucrose be added.

The composition could be in a dried form, preferably lyophilized or in aqueous solution before or after drying. The dried product is reconstituted with sterile water for injection or a buffer solution.

25

The claimed composition can also be a stable aqueous solution ready for use.

- 30 The invention also relates to compositions in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein, preferably more than 14 000 IU / mg.

- 35 The claimed composition can be prepared by mixing factor VIII with a non-ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt or by eluting factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

The invention also relates to the use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer
5 for a composition comprising coagulation factor VIII.

An amino acid is used to buffer the system and it protects also the protein in the amorphous phase. A suitable buffer could be L-histidine, lysine and/or arginine. L-Histidine has primarily been chosen because of the good buffer
10 capacity of L-histidine around pH 7.

Sucrose or sugar alcohol can also be added for the protection of the protein.

Calcium (or divalent metal ions), here added as calcium chloride (CaCl_2) but
15 other salts such as calcium gluconate, calcium glutubionate or calcium gluceptate can also be used, is necessary for the maintenance of the association of factor VIII heavy and light chain.

The data presented in the examples indicate that r-VIII SQ is stable for at
20 least 12 months when stored at $5 \pm 3^\circ\text{C}$.

The following examples illustrate the invention and show stability data for different formulations, all falling under the patent protection, a protection which is not limited to these examples.
25

The following figures are illustrating the invention:

Figure 1 HPLC gelfiltration, Example 10A, stored 5 months at 25°C .
Figure 2 HPLC gelfiltration, Example 10B, stored 5 months at 30°C .

EXPERIMENTAL

Material and methods

5 The production of recombinant factor VIII SQ (r-VIII SQ) was essentially performed as described in patent WO 91/09122, example 1-3. A DHFR deficient CHO celline (DG44N.Y.) was electroporated with an expression vector containing the r-VIII SQ gene and an expression vector containing the dihydrofolate-reductase gene. Following selection on selective media
10 surviving colonies were amplified through growth in stepwise increasing amounts of methotrexate. Supernatant from the resulting colonies were individually screened for VIII:C activity. A production clone was chosen and this was subsequently adapted to serum free suspension growth in a defined medium and finally a large scale fermentation process was developed.
15 Supernatant is collected after certain time periods and further purified as described below.

The clarified conditioned medium was pH adjusted and applied to a S-Sepharose FF column. After washing, factor VIII was eluated with a salt
20 buffer containing 5 mM CaCl_2 .

Immunoabsorption was carried out on an immunoaffinity resin where the ligand was a monoclonal antibody (8A4) directed towards the heavy chain of Factor VIII. Before loading to the column the S-eluate was treated with 0,3
25 % TNBP and 1 % Octoxynol 9.

The column was equilibrated, washed and factor VIII was eluated with a buffer containing 0,05 M CaCl_2 and 50 % ethylene glycol.

The mAb-eluate was loaded on a Q-Sepharose FF column, equilibrated with the elution buffer in the immunoaffinity step. After washing, factor VIII was
30 eluated with 0,05 M L-histidine, 4 mM CaCl_2 , 0,6 M NaCl, pH 6,8.

The Q-eluate was applied to a gel filtration column (Superdex 200 p.g.). Equilibration and elution was carried out with a formulation containing
35 sodium chloride, L-histidine, calcium chloride and polysorbate 80.

The protein peak was collected and the solution was formulated before freeze drying.

- 5 The VIII:C activity and the concentration of the inactive components were adjusted by diluting with an appropriate buffer. The solution was then sterile filtered (0,22 μ m), dispensed and freeze-dried. Samples from each composition were frozen and stored at - 70 °C. These samples were thawed and used as references during the assay of VIII:C.
- 10 The coagulant activity VIII:C was assessed by a chromogenic substrate assay (Coatest Factor VIII, Chromogenix AB, Mölndal, Sweden). Activated factor X (Xa) is generated via the intrinsic pathway where factor VIII:C acts as cofactor. Factor Xa is then determined by the use of a synthetic chromogenic substrate, S-2222 in the presence of a thrombin inhibitor I-2581 to prevent
- 15 hydrolysis of the substrate by thrombin. The reaction is stopped with acid, and the VIII:C, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 450 nm against a reagent blank. The unit of factor VIII:C is expressed in international units (IU) as defined by the current International Concentrate Standard (IS) established by
- 20 WHO.

The recovery of VIII:C is calculated as the percentage of VIII:C in the reconstituted solution divided by the VIII:C in the frozen and thawed solution for freeze-drying with appropriate adjustment for dilutions.

- 25 Soluble aggregates were determined by gel filtration. A prepacked Superdex 200 HR 10/30 column (Pharmacia) was used with a fluorescence detector (excitation wavelength 280 nm), emission wavelength 340 nm). The reconstituted preparation were analysed. Evaluation of results from
- 30 gelfiltration was done by visual examination of the chromatograms, or by integration of the peak areas if aggregates were found.

Recovery over freeze drying is expressed in % yield of frozen reference.

Example 1. Comparison between albumin and non-ionic surfactant.

Recombinant factor VIII was prepared according to the method described under Experimental.

5

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

The compositions were the following :

10		1 A	1B	1 C	1D
	L-Histidine, mM	50	50	50	50
	Sodium chloride, M	0,6	0,6	0,6	0,6
	Calcium chloride, mM	4	4	4	4
	Polysorbate 80, %	-	-	0.02	-
15	PEG 4000, %	0.1	0.1	-	-
	Albumin, %	-	1	-	1
	VIII:C charged IU/ml	250	250	250	250
	Recovery, IU/ml				
20	after reconstit.	83	197	232	222

This example shows that there was no difference in the recovery of factor VIII:C when the non ionic surfactant or albumin was used.

Example 2. Comparison between different strengths of non ionic surfactant

Recombinant factor VIII was prepared according to the method described under Experimental.

5

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 2 ml of sterile water for injections.

The compositions were the following :

10

	2 A	2B	2 C
L-Histidine/L-Glutamate equimolar amount, mg/ml	10	10	10
Sodium chloride, %	2	2	2
15 Calcium chloride, mg/ml	0.1	0.1	0.1
Polysorbate 80, %	-	0,001	0,01
VIII:C charged IU/ml	300	300	300
Recovery, IU/ml after reconstit.			
20 Initial	69	133	228
3.5 h*	43	140	222
7h*	49	133	204

* stored as reconstituted solution at ambient temperature

25

It is here clearly shown the surprisingly good stabilizing effect on factor VIII when a non ionic surfactant is used.

Example 3, Variation of non-ionic surfactant concentration.

Recombinant factor VIII was prepared according to the method described under Experimental.

5

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	3 A	3B	3 C	3D	3E
10 L-Histidine, mM	50	50	50	50	50
Sodium chloride, M	0.34	0.34	0.34	0.34	0.34
Calcium chloride, mM	4	4	4	4	4
Polysorbate 80, %	0.01	0.02	0.03	0.04	0.05
15 Recovery, after reconstit., %	91	90	93	99	100

Results from this example indicate that the recovery of factor VIII (VIII:C) was very high after reconstitution and good for all concentrations of polysorbate 80 used.

20

Example 4. Variation of sodium chloride concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

5

Two ml of the solution was lyophilized, stored at different temperatures for up to 6 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

10		4 A	4B
	L-Histidine.mM	50	50
	Sodium chloride, M	0.3	0.6
	Calcium chloride, mM	4	4
	PEG-4000 %	0.1	0.1
15	(Polyethylene glycol)		
	Polysorbate 80, %	0.025	0.025
	Recovery , %, initial	85	86
	stored at 8°C		
	3 mon	88	87
20	4 mon	87	83
	6 mon	87	83
	stored at 25°C, 1 mon	92	93
	3 mon	87	79
	4 mon	84	81
25	6 mon	85	85
	stored at 37°C 1 mon	88	90
	3 mon	80	80
	4 mon	80	77
	6 mon	81	80
30	stored at 50°C 1 mon	84	89
	3 mon	77	77
	4 mon	73	70

0,3 or 0,6 M sodium chloride showed very good stability. Both formulations were stable for 6 months at 37°C.

35

Example 5. Variation of L-Histidine concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

5

2,2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

10		5 A	5B
	L-Histidine, mM	46	59
	Sodium chloride, M	0.31	0.31
	Calcium chloride, mM	3,7	3,7
	PEG-4000 %	0.091	0.091
15	(Polyethylene glycol)		
	Polysorbate 80, %	0.364	0.364
	Recovery, %		
	stored at 8°C, Initial	78	84
	3 mon	70	76
20	stored at 25°C, 1 mon		
	3 mon	69	74
	stored at 37°C 1 mon	76	85
	3 mon	61	48
	stored at 50°C 1 mon	60	73
25	3 mon	44	48

This example shows that these different amounts of L-histidine does not effect the stability.

30

Example 6

Recombinant factor VIII was prepared according to the method described under Experimental.

5

	6A	6B
L-Histidine, mM	65	65
Sodium chloride, M	0.3	0.3
Calcium chloride, mM	4	4
10 PEG-4000 %	0	0.1
Tween 80, %	0.025	0.025

These solutions were freezed/thawed 1, 5 and 10 times and the recovery was the following:

15

	IU/ml	IU/ml
cold	298	291
1 freezing	293	293
5	295	287
20 10	290	288

These studies showed that VIII:C was stable after repeated freeze-thawing and that PEG-4000, which is thought to act as cryoprotectant, is not necessary in this formulation.

Example 7. Variation of pH

Recombinant factor VIII was prepared according to the method described under Experimental.

5

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	7 A	7B	7 C	7D
10 L-Histidine, mM	65	65	65	65
Sodium chloride, M	0,3	0,3	0,3	0,3
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	0.025	0.025	0.025	0.025
pH	6.0	6.5	7.0	7.5
15 Recovery, %, Initial	74	70	78	79
3 hours*	73	80	78	77

*stored as reconstituted solution at ambient temperature

20 This example shows that a pH is of no significant importance between 6.0 and 7.5 approx.

Example 8 Addition of sucrose

Recombinant factor VIII was prepared according to the method described under Experimental.

25

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	8A	8B
30 L-Histidine, mM	58	20.5
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	3,7	3,7
Sucrose, mM	0	13.3
Polysorbate 80, %	0.025	0.025

35

Sucrose was added to the solution B after the final purification step before lyophilization.

The recovery after freeze-drying was 76 % for A and 87 % for B. The same activity was found 4 hours after reconstitution stored at room temperature.

- 5 This study indicated that the addition of sucrose is favourable for the recovery of VIII:C over freeze-drying.

Example 9 . Variation of calcium salt

- 10 Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

15		9 A	9B	9C	9D
	L-Histidine, mM	23	23	23	23
	Sodium chloride, M	0,34	0,34	0.34	0,34
	Calcium chloride, mM	4	4	0.15	0.15
	Polysorbate, %	0.025	0.025	0.025	0.025
20	Sucrose, mM	-	10	-	10
	Calciumgluconate, mM	0	0	6	6
	Recovery, %, Initial	63	74	74	78
	4 hours*	60	73	73	77

- 25 *stored as reconstituted solution at ambient temperature

This example shows that CaCl₂ can be substituted by Calcium gluconate.

Example 10

Recombinant factor VIII was prepared according to the method described under Experimental.

5

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 1000 IU.

10		10A	10B
	L-Histidine, mM	14.7	58
	Sodium chloride, M	0.31	0.31
	Calcium chloride, mM	3.7	3.7
	Sucrose, mM	19.9	-
15	Polysorbate 80, %	0.025	0.025
	Recovery, IU/ml after reconstitution		
	Initial	213	198
	4 h, 25 °C	213	198
20	24, 25 °C	201	182
	Recovery, %		
	Initial	92	91
	5 months, 25°C	88	-
	5 months, 30°C	76	85
25	12 months, 7°C	89	97

The recovery was good when part of the L-histidine was substituted by sucrose.

30 These formulations were studied by gelfiltration after 5 months' storage at 25°C and 30°C, respectively and the results are shown in figures 1 and 2. The only peaks to be seen is the peak at 42, indicating factor VIII:C and the peak at 70 which is histidine. Aggregates is to be found earlier than 40. From figure 1 it can be seen that no detectable amount of aggregates was found after 5 months at 25°C for 10A. Figure 2 shows a small amount of
35 aggregates which is less than 2 % after 5 months at 30°C for 10B.

SUBSTITUTE SHEET

Example 11

Recombinant factor VIII was prepared according to the method described under Experimental.

5

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

10

		11A	11B
	L-Histidine, mM	14.7	58
	Sodium chloride, M	0.31	0.31
	Calcium chloride, mM	3.7	3.7
15	Sucrose, mM	19.9	-
	Polysorbate 80, %	0.025	0.025
	Recovery, IU/ml after reconstitution		
	Initial	98	105
20	4 h, 25 °C	96	103
	24, 25°C	93	101
	Recovery, %		
	Initial	91	93
	stored at 25°C, 5 mon	89	87
25	stored at 30°C, 5 mon	76	79
	stored at 7°C 12 mon	88	89

Both formulations showed good stability.

These formulations were studied by gelfiltration and the results were similar as shown in Figures 1 and 2.

30

No aggregation was formed when the formulations had been stored for 5 months at 25°C and 30°C, respectively.

Example 12

Recombinant factor VIII was prepared according to the method described under Experimental.

- 5 2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 4 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

10

	12A	12B
Mannitol, mg/ml	20	20
L-Histidine, mg/ml	2,67	2.67
Sodium chloride, mg/ml	18	18
15 Calcium chloride, mM	3,7	3,7
Polysorbate 80, mg/ml	0.23	0.23
Recovery, %		
initial	91	93
stored at. 7°C 5 mon	90	85

20

An acceptable stability was achieved after five months at 7°C.

CLAIMS

- 5
1. A composition comprising coagulation factor VIII and a non-ionic surfactant as stabilizer.
 2. A composition according to claim 1 in which factor VIII is highly purified
10 and stable without the addition of albumin.
 3. A composition according to claim 1 or 2 in which factor VIII is full-length or a deletion derivative of recombinant factor VIII.
 - 15 4. Composition according to any of claims 1-3 in which the amount of factor VIII is 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.
 5. Composition according to any of claims 1-4 in which the non-ionic surfactant is present in an amount above the critical micelle concentration.
20
 6. Composition according to any of claims 1-5 in which the non-ionic surfactant is chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80.
 - 25
 7. Composition according to claim 6 in which the polyoxyethylene (20) fatty acid ester is in an amount of at least 0.01 mg/ml.
 8. Composition according to any of claims 1-7 which comprises sodium or
30 potassium chloride, preferably in an amount of more than 0.1 M.
 9. Composition according to any of claims 1-8 which comprises calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
 - 35
 10. Composition according to any of claims 1-9 which comprises an amino acid such as L-histidine in an amount of more than 1 mM.

11. Composition according to any of claims 1-10 which comprises mono-or disaccharides, preferably sucrose or sugar alcohols.
- 5 12. Composition according to any of claims 10-11 which comprises L-histidine and sucrose.
13. Composition according to claim 8 and 10 in which the ratio sodium chloride to L-histidine is more than 1:1.
- 10 14. Composition according to any of claims 1-13, comprising
- i) 10-100 000 IU/ml of recombinant factor VIII
 - ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
 - iii) sodium chloride, preferably in an amount of more than 0.1 M.

15 iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.

v) an amino acid such as L-histidine in an amount of more than 1 mM.
15. Composition according to any of claims 1-14 which is dried.
- 20 16. Composition according to claim 15 which is lyophilized.
17. Composition according to any of claims 1-14 which is in a stable aqueous solution ready for use.
- 25 18. Composition according to any of claims 3-17 in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.
- 30 19. Process for the preparation of the composition according to claim 1 characterized by mixing factor VIII with a non ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.
- 35 20. Process for the preparation of the composition according to claim 1 characterized by eluting factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqueous solution, preferably

23

together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

21. Use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

10

1/2

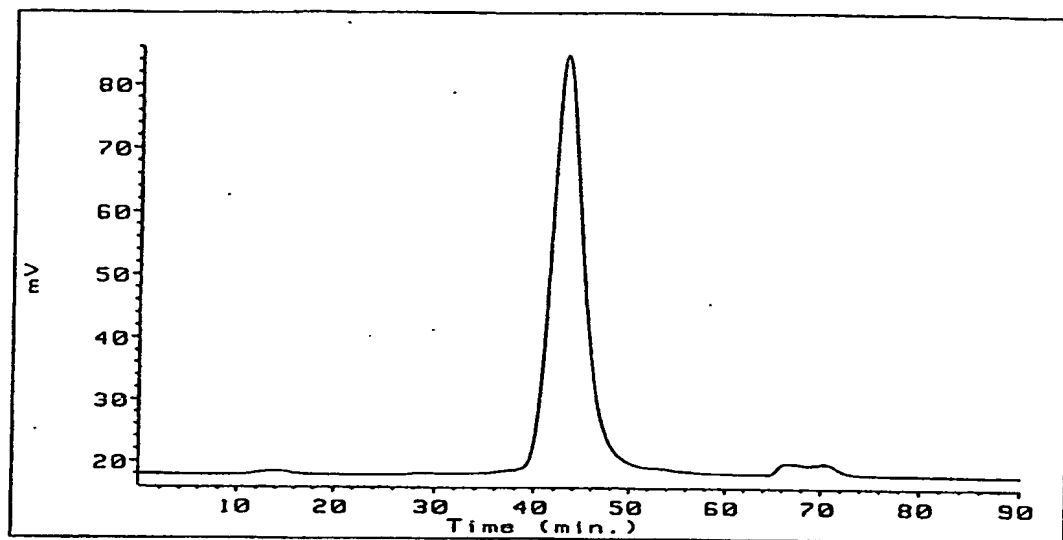


Figure 1

SUBSTITUTE SHEET

2/2

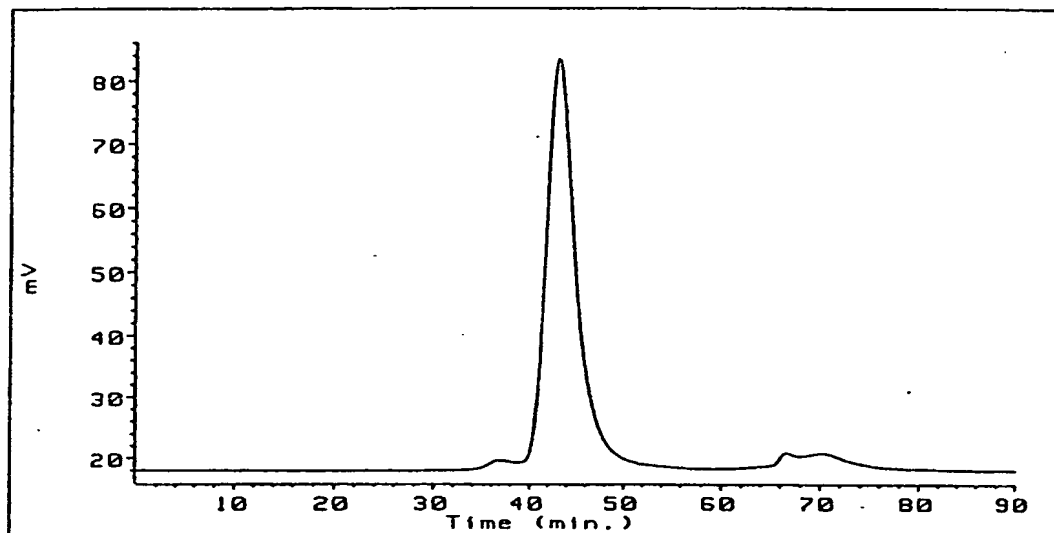


Figure 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00793

A. CLASSIFICATION OF SUBJECT MATTER		
IPC5: A61K 35/16, A61K 37/02 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC5: A61K, C07K, C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP, A1, 0508194 (BEHRINGWERKE AG), 14 October 1992 (14.10.92), see claim 6, examples 1-2 ---	1-21
X	EP, A3, 0099445 (NEW YORK BLOOD CENTER, INC.), 1 February 1984 (01.02.84), see page 8, line 7 - line 14; page 19, line 24 - page 20, line 27 ---	1-21
A	WO, A1, 9110439 (OCTA PHARMA AG), 25 July 1991 (25.07.91) -----	1-21
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"B" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search		Date of mailing of the international search report
4 January 1994		12 -01- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/11/93

International application No.

PCT/SE 93/00793

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0508194	14/10/92	AU-A- 1470292 DE-A- 4111393 JP-A- 5097702	15/10/92 15/10/92 20/04/93
EP-A3- 0099445	01/02/84	SE-T3- 0099445 AU-B- 561900 AU-A- 1346283 CA-A- 1207229 JP-A- 58222023 US-A- 4481189 US-A- 4591505	21/05/87 20/10/83 08/07/86 23/12/83 06/11/84 27/05/86
WO-A1- 9110439	25/07/91	DE-A- 4001451 EP-A- 0511234	01/08/91 04/11/92

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.